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Isolation of long-chain aliphatic alcohols from beeswax using lipase-catalyzed methanolysis in supercritical carbon dioxide

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Abstract

Aliphatic alcohols of chain lengths of 24–34 carbons have been found to be beneficial in treating hypercholesterolemia. Approximately 40% of beeswax is long-chain esters which can be transesterified in supercritical carbon dioxide to give these alcohols and fatty acid methyl esters. The methanolysis reaction was catalyzed by an immobilized lipase from *Candida antarctica* in flowing supercritical carbon dioxide and the alcohols were then isolated from the fatty acid methyl esters by precipitation from heptane. The alcohols were found in the following percentages: tetracosanol, 9.1; hexacosanol, 14.0; octacosanol, 18.4; triacontanol, 37.2; dotriacontanol, 21.1; tetratriacontanol, 0.3. The utility of the method was demonstrated by isolating fatty alcohols from jojoba oil and a mixture of triglycerides and waxes isolated from corn bran.
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1. Introduction

There has been recent interest in the long-chain aliphatic alcohols as dietary supplements or nutraceuticals in the treatment of various chronic diseases including diabetes and hypercholesterolemia. General reviews of the medicinal claims for the use of these compounds—commonly known as policosanols—have been published [1,2]. Several studies have reported on the effectiveness of policosanols in lowering total cholesterol and the "bad" low density lipoprotein, while increasing the "good" high density lipoprotein by inhibiting cholesterol synthesis and increasing metabolism of low density lipoprotein [3–7]. These effects are brought about without apparent side effects [7–9]. Controversy over the effectiveness of these compounds remains, however [10], in that Lin et al. found no effect on blood cholesterol levels in healthy volunteers placed on regimen of wheat germ policosanols. Although policosanols are available from various sources, most medical studies have used policosanols isolated from sugar cane where they make up 0.1–0.3% of the mass of the canes.

Beeswax of the honey bee (*Apis mellifera*) is a complex mixture of long-chain alkanes, alkenes, monoesters, diesters, hydroxymonoesters, and fatty acids as well as several minor components [11]. Much of the ester fraction contains palmitic, oleic, and tetracosanoic acid esters of alcohols ranging in length from 12 to 36 carbons [12]. Monoesters of C_{24} to C_{34} alcohols are about 40% of the composition of beeswax. Saponification of these esters yields the long-chain alcohols of interest. Annual production of beeswax in the United States is about 1 million kilograms making this wax an attractive source of policosanols.

At least four United States patents have been issued for the isolation of these alcohols [13–16]. These patents claim to isolate the alcohols directly or after saponification and all use organic solvents, such as heptane, acetone, toluene, benzene, phenol, methanol, trichloroethane, and chloroform in the process. Supercritical carbon dioxide (scCO$_2$) has emerged as a preferred solvent in the trend toward "green" chemistry [17,18]. It is environmentally benign, inexpensive, nontoxic, and readily available. It is also well known as a good solvent for lipids and lipid-like materials. Manipulation of scCO$_2$ is easy in that product isolation can often be accomplished simply by depressurization, allowing product to "fall out" of the fluid stream. We report here a method for isolating these alcohols by transesterification of the beeswax esters in scCO$_2$ without the need for the large volumes of organic solvents.

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2. Experimental

2.1. Materials

Beeswax was from a local apiary housing *A. mellifera* in an area where the forage crops were largely soy beans (*Glycine max*), pumpkins (*Cucurbita moschata*), and wildflowers. The wax was collected from the cappings after honey harvest. The wax was cleaned of remaining honey and hive debris by melting in enough hot water to float the wax. Upon cooling, the wax was removed and the process repeated two more times. It was not further refined. HPLC grade Methanol was from Fisher Scientifics (Fairlawn, NJ). Novozym 435 was from Novozymes North America (Franklinton, NC). Activity of the immobilized enzyme was stated to be 12,400 propyl laurate per gram. Linear alcohols of 24, 26, 28, and 30 carbons and alumina (Brockman type 1, neutral, not further activated), were from Sigma–Aldrich (Milwaukee, WI). Jojoba oil was from Floratech Americas (Gilbert, AZ). The corn bran extract was a by-product of a previous research project [19].

2.2. Methods

The reactions were carried out in an apparatus previously described [20]. The system was modified such that three cells were connected in series, see Fig. 1. The first cell was charged with beeswax, the second contained Novozym 435, and the third held alumina. Methanol was pumped into the system at 0.20–0.50 ml/min using a solvent pump (Applied Separations Speed Max Model 10000). Carbon dioxide flow was maintained using a micrometering valve located inside the heated apparatus. Products were collected in a 500 ml flask after expansion of carbon dioxide. The alumina was washed by increasing methanol flow to 0.5 ml/min and collecting products in a new flask.

A rough solubility of beeswax was determined by placing a 5 g piece of wax (about $1 \, cm \times 1 \, cm \times 2 \, cm$) in a cell and passing scCO$_2$ through the system. Fractions were collected and weighed at various times. The mass of material collected per mass of CO$_2$ used gave the weight percent solubility.

2.3. Chromatography

Mass spectra were collected using an Agilent Technologies (Palo Alto, CA, USA) Model 6890 Network GC and an Agilent 5973 Network mass selective detector. Separations were accomplished using a 30 m DB-5HT narrow bore column (Agilent Technologies) heated from 100 °C to 380 °C at 10 °C/min. The carrier gas was helium. Integration results are reported as GC area percentages.

2.4. Methanolysis

In a typical conversion, about 5.11 g beeswax were placed in the first cell, 40 g Novozym 435 were placed in the second cell, and 10 g alumina were placed in the third cell. Carbon dioxide was pumped in and brought to 27.5 MPa using a Haskel booster pump (Haskel, Burbank, CA). The entire apparatus was heated to 70 °C and methanol was pumped in at a rate of 0.2 ml/min. Expanded gas flow was maintained at 5 l/min. This is a CO$_2$ density of 0.77 g/ml in the reactor and a methanol concentration of 1.6 wt%. After 1600 l CO$_2$ had passed, 5.40 g product had collected. A second receiver was put in place when collection of product in the first flask had decreased significantly indicating that the more soluble materials had been collected. The methanol flow was then increased to 0.5 ml/min. After another 350 l CO$_2$ had passed, 0.88 g products had collected. The first fraction was dissolved in warm heptane and then placed in a freezer at −20 °C until the alcohols precipitated. This precipitate was collected by filtration and washed with hexane and acetone to give 1.45 g alcohols (28% based on starting mass of wax).

2.5. Product analysis

Samples were dissolved in chloroform for collection of mass spectra (eims), M−H$_2$O: tetracosanol, 346; hexacosanol, 364; octacosanol, 392; triacontanol, 420; dotriacontanol, 448; tetratriacontanol, 476. These were compared to known samples when available.

The products washed from the alumina were dissolved in chloroform and were found unreacted esters as identified by their
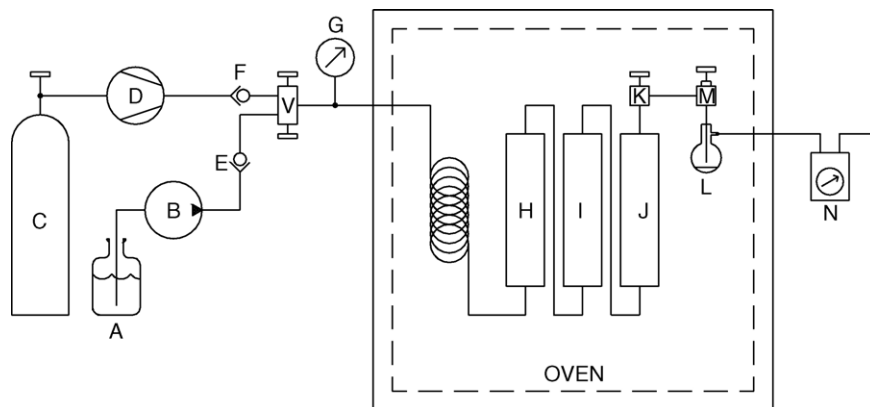

Fig. 1. Schematic of the system used for the methanolysis of waxes: (A) methanol source feeding; (B) the HPLC pump; (C) CO$_2$ source and (D) booster pump; (E) and (F) check valves; (G) pressure transducer, cells for wax; (H) Novozym 435; (I) and (J) alumina; (K) shut off valve; (L) receiver; (M) micrometering valve; (N) gas flow meter.

Table 1
Abundance of fatty acid methyl esters found after methanolysis of beeswax

Fatty acid	Found (%)
Palmitic	51
Tetracosanoic	13
Hexadecenoic	12
Oleic	5
Hexacosanoic	5
Octacosanoic	5
Triacontanoic	4
Dotriacontanoic	3
Tetratriacontanoic	3

mass spectra (eims), m/e (relative intensity): tetracosyl palmitate, 257 (100), 593 (15); hexacosyl palmitate, 257 (100), 621 (18); octacosyl palmitate, 257 (100), 649 (15); triacontyl palmitate, 257 (100), 677 (15); dotriacontyl palmitate, 257 (100), 705, (13); dotriacontyl oleate, 264 (100), 731 (3); tetratriacontyl palmitate, 257 (100), 733 (15). Jojoba oil products were identified as methyl oleate, 264 (100), 296 (19); 11-eicosenoic acid methyl ester, 292 (100), 324, (22); 13-docosenoic acid methyl ester, 320 (100), 352 (9); 11-eicosenol, M-18, 278; 13-docosenol, M-18, 306; tetracosenol, M-18, 334.

3. Results and discussion

Analysis of our beeswax sample by gas chromatography mass spectroscopy indicated that 47% of the wax was made up of esters, and these contain the C_{24}–C_{34} alcohols combined primarily with palmitic and oleic acids. Other components include the odd numbered alkanes from triacosane to triatriacontane, which make up 39% of the wax, with the remaining 14% made up of odd-numbered alkenes, hydroxyl esters, and branched alkanes. Fig. 2 includes the gas chromatogram of the starting beeswax. This analysis is from the 16 largest peaks in the chromatogram, and is not exhaustive, but is in general agreement with previously published work [11,12]. We used these results to analyze the beeswax as a starting material not intending to draw any new conclusions about beeswax from the analysis. The solubility of beeswax in supercritical CO_2 was found to be 0.2 wt% at our reaction conditions of 27.5 MPa and 70 °C. This is comparable to the solubility of wool wax esters [21]. However, the large number of compounds that constitute beeswax and other waxes complicate efforts to make these measurements.

The composition of beeswax indicated that it should be a good source of the long-chain alcohols that have garnered interest in recent years. Complete conversion of the esters to alcohols could give about 300 g/kg of wax. We chose to work in supercritical CO_2 to avoid the copious use of organic solvents as described in the patent literature. Though "green" technologies are most urgent for larger scale production, even a small-scale pharmaceutical preparation is more attractive if it can be accomplished with limited solvent use. We chose methanolysis owing to the miscibility of methanol in CO_2 and the effectiveness of Novozym 435 in catalyzing this conversion in supercritical CO_2. The products were collected as colorless, odorless solids in the receiver. GC/MS analysis of this mixture showed 22 peaks identifiable as the six alcohols, 10 fatty acid methyl esters, three alkanes, a C_{31} unsaturated hydrocarbon, and two unreacted esters. These peaks make up 91.6% of the of the integrated peak area with the remaining 8.4% of the area as unidentified peaks. Fig. 2 shows the gas chromatograms of the starting beeswax, initial product mixture, and final alcohol mixture. The product mixture collected upon depressurization of the CO_2 was found to be 41% alcohols in the following distribution: $C_{24}OH$, 15.1%; $C_{26}OH$, 16.6%; $C_{28}OH$, 18.7%; $C_{30}OH$, 30.6%; $C_{32}OH$, 17.9; $C_{34}OH$, 1.0%. The solid was then dissolved in heptane and then cooled to precipitate the alcohols at 90% purity and the following distribution: $C_{24}OH$, 9.0%; $C_{26}OH$, 13.9%; $C_{28}OH$, 18.3%; $C_{30}OH$, 36.9%; $C_{32}OH$, 20.8; $C_{34}OH$, 1.0%. The greater solubility of the shorter chain alcohols in hexane and heptane leads to the enrichment in the longer chain alcohols in the precipi-
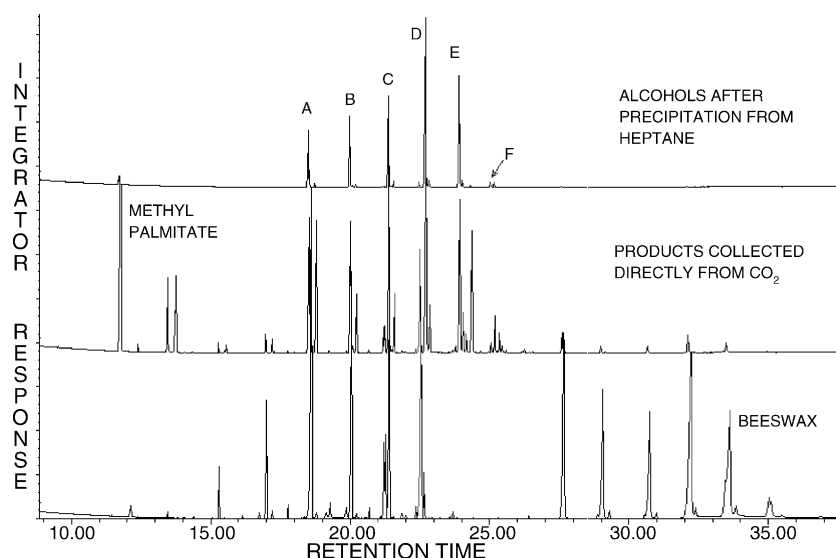

Fig. 2. GC/MS showing the methanolysis of beeswax from initial wax, bottom, to all products collected from expansion of CO_2, center, and alcohols after precipitation from heptane: (A) tetracosanol; (B) hexacosanol; (C) octacosanol; (D) triacontanol; (E) dotriacontanol; (F) tetratriacontanol.

tated product. This highlights a difficulty in isolating a class of compounds as opposed to a single component.

The fatty acid methyl esters formed from the methanolysis are shown with their relative quantities in Table 1. The predominant acid found was palmitic acid followed by tetracosanoic acid and hexenoic acid. The analysis of our starting beeswax found palmitic acid esters to be the largest source of aliphatic alcohols present.

Alumina was included in the carbon dioxide flow in order to remove unreacted esters and the brightly colored pigments in the wax from the product stream. Although the alumina was used directly from the bottle without further preparation, it retained a significant quantity of pigment, unreacted esters, alcohols, and fatty acid methyl esters, as well as the characteristic sweet fragrance of beeswax. This allowed for the collection of odorless and colorless products. All but the bright yellow pigment and fragrances could be washed from the alumina by increased methanol flow.

To demonstrate the utility of this method, we performed the methanolysis on jojoba oil and a mixture of triglycerides and waxes isolated from corn bran. Jojoba oil is composed of esters made of an eicosenoic acid fragment (71%) coupled with an eicosenol (45%) or docosenol (44%). The resulting waxes are then composed mainly of $C_{40:2}$ and $C_{42:2}$ esters [22]. Our sample analyzed as 48% $C_{42:2}$, 31% $C_{40:2}$, 11% $C_{44:2}$, and 8% $C_{38:2}$. These liquid waxes were poured onto a glass wool plug and the methanolysis performed in the same manner described for beeswax. Products found in the receiver included the methyl esters of 11-eicosenoic acid (76%), 13-docosenoic acid (15%), and oleic acid (7%). The alcohols were then washed from the alumina to give 11-eicosenol (41%), 13-docosenol (48%), and tetracosenol (10%) as the only products adsorbed to the alumina. This presents a second potential benefit of having alumina in the reaction stream: with enough alumina available, polar compounds like alcohols, adsorb to the alumina and can be separated from the less polar reaction products. This offers a chance to reduce organic solvents in the workup of products.

The corn bran material was obtained from a $scCO_2$ extraction of corn bran and consisted of a mixture of waxes and corn oil triglycerides. For the methanolysis, about 2 g of the mixture were poured onto a plug of glass wool in the extraction cell and the reaction performed as with beeswax. After 300 l CO_2 passed, receivers were changed and methanol flow increased to 0.5 ml/min. This first fraction contained methyl esters of fatty acids, mostly oleic and linoleic from the corn oil. After another 300 l CO_2 passed, a colorless product had collected. This mass was dissolved in warm heptane. Upon cooling, a precipitate of 96% pure alcohols was collected. The relative quantities of alcohols was found to be: $C_{24}OH$, 1.7%; $C_{26}OH$, 4.8%; $C_{28}OH$, 11.8%; $C_{30}OH$, 41.1%; $C_{32}OH$, 37.7; $C_{34}OH$, 2.9%.

4. Conclusion

Waxes were shown to be readily converted to their respective alcohols and fatty acid methyl esters by lipase-catalyzed methanolysis. Immobilized Novozym 435 was found to be effective in the conversion without any apparent loss of activity after several runs. The fatty alcohols from beeswax were isolated in the following ratios: $C_{24}OH$, 9.0%; $C_{26}OH$, 13.9%; $C_{28}OH$, 18.3%; $C_{30}OH$, 36.9%; $C_{32}OH$, 20.8; $C_{34}OH$, 1.0%. Whereas, corn bran fatty alcohols were found in the following ratios: $C_{24}OH$, 1.7%; $C_{26}OH$, 4.8%; $C_{28}OH$, 11.8%; $C_{30}OH$, 41.1%; $C_{32}OH$, 37.7; $C_{34}OH$, 2.9%. The corn bran waxes have higher quantities of the longer chain alcohols compared to the composition of beeswax. The addition of alumina in the reaction stream was found to aid in the clean-up of products by adsorbing highly colored pigments from wax and by retaining unreacted esters. In clean waxes, such as those in refined jojoba oil, the alumina was found to retain alcohols allowing for the separation of alcohols from methyl esters without the use of organic solvents, adding to the credentials of $scCO_2$ as a green medium.

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